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Post-transcriptional gene silencing in plum pox virus resistant transgenic European plum containing the plum pox potyvirus coat protein gene

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Abstract

Transgenic plums containing the plum pox potyvirus coat protein (PPV-CP) gene were inoculated with PPV. Infection was monitored by evaluating symptoms, ELISA, and IC-RT-PCR. Transgenic clone C5 was highly resistant to PPV during four years of testing and displayed characteristics typical of post-transcriptional gene silencing (PTGS), including a high level of transgene transcription in the nucleus, low levels of transgene mRNA in the cytoplasm, a complex multicopy transgene insertion with aberrant copies, and methylation of the silenced PPV-CP transgene. The PPV-CP transgene was also methylated in seedlings of C5 and these seedlings were resistant to PPV. Our results show, for the first time, that PTGS functions as a mechanism for virus resistance in a woody perennial species.

Introduction

Virus diseases of tree fruits, including plum pox or sharka virus (PPV) of *Prunus*, cause extensive economic losses (Németh, 1994). Control of tree fruit virus diseases has been through the control of insect or nematode vectors, sanitation and quarantine. While the use of resistant germplasm for developing virus resistant cultivars is a preferred method of control, there are few, if any, sources of resistance to most tree fruit viruses. The transformation of plants with viral genes, such as coat protein (CP), can provide novel virus resistant varieties or gene resources for breeding new resistant varieties. Transgenic plants expressing viral genes have been shown to exhibit varying degrees of resistance to viruses homologous or closely related to the source of the transgene (Beachy et al., 1990). Studies of viral transgene-mediated resistance have shown that resistance may either be mediated through the production of transgene protein (Wilson, 1993) or RNA-mediated. RNA-mediated resistance may take the form of post-transcriptional gene silencing (PTGS) wherein mRNA is degraded in the cytoplasm soon after synthesis (Dehio & Schell, 1994; Tanzer et al., 1997). PTGS has been associated with multiple transgene copies, particularly direct repeats of the transgene coding region (Dehio & Schell, 1994; Sijen et al., 1996), truncated or antisense copies of the transgene insert (Stam et al., 1997; Waterhouse et al., 1998; Kohli et al., 1999), methylation of the coding region (Ingelbrecht et al., 1994; English et al., 1996; Davies et al., 1997; Guo et al., 1999; Jones et al., 1999;

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Kohli et al., 1999; Sonada et al., 1999), a low level of RNA accumulation in the cytoplasm, and RNA accumulation in the nucleus higher than or equal to susceptible transgenics (Dehio & Schell, 1994; Kunz et al., 1996; Tanzer et al., 1997). PTGS may require 'meiotic resetting' or reinitiation of the mechanism after each generation (Dehio & Schell, 1994; Kunz et al., 1996; Balandin & Castresana, 1997; Tanzer et al., 1997). Grafting experiments have shown that PTGS plants may produce a systemic signal that can interact with homologous native genes or transgenes to produce the PTGS state. Non-PTGS plants containing sequences homologous to the PTGS sequence can become silenced when grafted onto a PTGS stock but revert to a non-silenced state when they are removed from the PTGS stock and are grafted onto non-PTGS plants (Palauqui et al., 1997; Palauqui & Vaucheret, 1998).

These observations have formed the basis for several models that have been extensively reviewed and discussed (Dougherty & Parks, 1995; Baulcombe, 1996; Sijen et al., 1996; Wassenegger & Pélissier, 1998; Depicker & Van Montagu, 1997; Carrington & Whitham, 1998).

Most of the research on viral gene-mediated protection, and essentially all of the work on PTGS have been reported from herbaceous systems. To our knowledge, PTGS has not been reported in woody perennial plant species. We previously reported the development of transgenic plums expressing *PPV-CP* genes. Plum trees with different transgene copy numbers were produced (Scorza et al., 1994). In particular, transgenic clone C5, which contained a multicopy transgene insertion, produced a low level of CP mRNA and no detectable CP while other clones that contained single or multiple copies produced both mRNA and CP. When inoculated with PPV clone C5 was highly resistant (Ravelonandro et al., 1997). The multicopy nature of the insert in C5, the low level of accumulated CP mRNA and the lack of CP protein suggested a PTGS mechanism of resistance.

In this paper we report on the mechanism of PPV resistance shown by transgenic clone C5. Nuclear transcription assays demonstrated that transcription rates of transgene PPV-CP in C5 were high. We found that clone C5 contains a complex arrangement of transgene copies that includes the entire construct cassette and aberrant copies. The PPV-CP transgene insert in this line is methylated. Seedlings carrying the C5 insert, like the C5 parent, displayed CP transgene methylation, and exhibited a high level of resistance to PPV. Our results show for the first time, to our knowledge, that PTGS functions to induce and maintain virus resistance in a woody perennial.

Materials and methods

Transgenic plum lines

Transgenic plum clones C3–C6 used in this study were described previously (Scorza et al., 1994; Ravelonandro et al., 1997). Clone PT6 was produced in the same manner as the C-series of clones. It contains two copies of the CP insert, expresses moderate levels of CP-mRNA and CP. Clone PT23, derived from transformation with the plasmid pGA482GG (Fitch et al., 1990; Ling et al., 1991), contains the vector marker genes without the PPV-CP insert.

Plant culture conditions

Buds from transgenic plants were grafted onto seedling rootstocks derived either from 'Rutgers Redleaf' peach (*P. persica*) or 'Bluebyrd' plum (*P. domestica*). Grafted plants were cultured in a greenhouse maintained at 22–24°C with an 18-h day length provided by a mix of natural and supplemental high intensity lighting. To provide cold-induced dormancy (CID), plants were pruned to approximately 0.75 m in height, leaves were removed and plants were maintained at 5°C, 15-h dark, 9-h low light/24 h for two months in a growth chamber (CONVIRON, Asheville, NC).

Inoculation with PPV

Each test plant was inoculated five to eight months following the initial graft of the transgenic plum onto peach or plum rootstock by grafting two buds from a *P. tomentosa* infected with PPV strain M obtained from INRA (Bordeaux, France). One bud was inserted into the transgenic scion and the other into the non-transformed rootstock. Infection of the inoculum source was verified by ELISA of each budstick from which inoculum buds were excised. A total of 6–9 inoculated plants per clone were produced (except in the case of PT6-1 where four plants were inoculated). Inoculum buds were allowed to grow and produce shoots on inoculated plants. The shoots that developed from these inoculum buds were evaluated for symptoms, and if none were visible, ELISAs were performed. When root suckers (adventitious shoots from roots) developed from the seedling plum rootstocks (root suckers do not develop from peach rootstocks)

these were evaluated for infection through symptom expression or by ELISA.

Visual evaluation of PPV symptoms

The development of visual symptoms of PPV infection, as described by Damsteegt et al. (1997), was periodically monitored beginning at 30 days post inoculation. Symptoms were scored on a scale of zero (no symptoms) to four (severe, including leaf necrosis).

Bio-evaluation of PPV

In order to evaluate the presence of PPV in symptomless or ELISA negative plants, two buds each from healthy, non-transformed, PPV susceptible *P. tomentosa* and *P. domestica* were grafted onto test plants 21 months post-inoculation. Shoots were allowed to grow from these buds. Evaluation of PPV symptoms and ELISA were carried out on leaves from these shoots.

ELISA detection

Leaves from inoculum sources and from inoculated plants were assayed by indirect triple-antibody sandwich TAS-ELISA (Ravelonandro et al., 1997). ELISA did not detect the presence of transgene-produced PPV-CP in any of the non-PPV-inoculated PPV-CP transgenic clones.

DNA isolation

Leaves for DNA extraction were collected and frozen in liquid nitrogen. DNA was extracted using a 10-fold scale-up of the procedure described by Kobayashi et al. (1998) with the addition of an RNase A treatment (Maniatis et al., 1982). RNA for RT-PCR was extracted using a PureScript RNA isolation kit (Gentra Systems Inc., Minneapolis, MN).

DNA hybridization

Restriction digestions were performed using 10 units of enzyme to digest 10 μg of DNA in the appropriate reaction buffer at 37°C. Agarose gels ranging from 0.8 to 1.4% were used to resolve fragments depending on expected fragment sizes. DNA was transferred to membranes and hybridized as per manufacturer's directions using the chemiluminescent digoxigenin (DIG) detection system (Roche Molecular Biochemicals, Indianapolis). Hybridization probes were generated by incorporating DIG-dUTP, as per manufacturer's directions during PCR, using gene-specific primers with plasmid pGA482GG/PPV-CP-33 (Scorza et al., 1994) as a template. Probes for DNA hybridization in Figure 6 were produced as described by Scorza et al. (1995). The primers and PCR conditions used to generate the 1.0 kbp PPV-CP fragment were described by Scorza et al. (1994). The 1.1 kbp *npt*II and 0.8 kbp *gus*A fragments were described by Scorza et al. (1995). The primer sequences for the CaMV 35S promoter probe were (5′) 5′-GATGGTTAGAGAGGCTTACGC-3′ and (3′) 5′-CGCAATGATGGCATTTGTAGG-3′.

Polymerase chain reaction (PCR)

PCR to evaluate methylation was performed on the digested DNA used for the DNA-blot of Figure 6, employing the same primers and reaction conditions for *npt*II, *gus*A, PPV-CP, and CaMV 35S as were used for producing the hybridization probes. In order to obtain a similar level of product formation, template quantities required for C5 and C3 were 50 and 100 ng, respectively.

Reverse transcriptase (RT)- and immunocapture (IC)-PCR

RT-PCR was conducted as previously described (Levy & Hadidi, 1994) using PPV-specific primers to the 3′ non-coding region and using 0.7 μg total RNA per reaction. For immunocapture (IC-RT-PCR), virus particles were captured in microfuge tubes pre-coated with a 1:200 dilution of anti-PPV polyclonal antisera (BIOREBA, Inc., Carrboro, NC) prior to amplification.

Isolation of nuclei and nuclear run-on transcription assays

Protoplasts were isolated from young leaves of greenhouse-grown plum plants using a modification of the procedure of Saxena et al. (1985) and verified using 4′, 6′-diamidino-2 phenylindole (DAPI). Nuclei were released in buffer, and the production of labeled nascent nuclear RNAs was based on 1.5×10^5 nuclei per reaction (Dehio & Schell, 1994; Pang et al., 1996). Nuclear run-on transcripts from non-transformed B70146 plum and transgenic C4 and C5 plants were hybridized to a blot of restriction enzyme-digested actin and PPV-CP cDNA plasmids.

Results

Transgenic clone C5 is symptomless and PPV ELISA negative

In order to evaluate the resistance of PPV-CP transgenic clones, PPV symptoms and ELISA were evaluated.

In the July of the first test year, approximately one month post-cold-induced dormancy (CID) (nine months post-inoculation), PPV symptoms were only visible on control plants. At 12 months post-CID and beyond, the majority of the transgenic clones showed symptoms except those of transgenic clone C5 which did not show symptoms of PPV at any time during the course of the study.

Leaves were sampled for ELISA at the time of symptom evaluation. At one month post-CID (nine months post-inoculation) all plants of transgenic clones PT6 and C5 were ELISA negative (Figure 1). All other transgenic clones were positive for PPV. Three months later, all clones except C5 were ELISA positive. Plants of clone C5 remained ELISA negative while clone PT6 had a low level or no ELISA-detectable infection throughout the year sampling period. Periodic ELISA sampling for two additional years failed to detect PPV in clone C5.

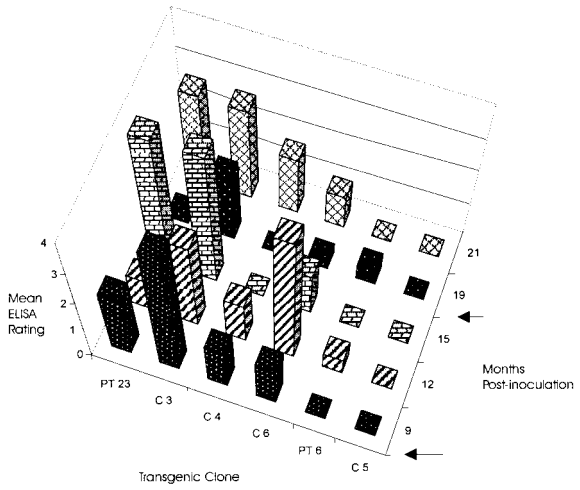

Figure 1. TAS-ELISA evaluation of PPV infection of PPV-CP transgenic plum clones. A control clone containing the transgene without the PPV-CP insert (PT23) is included. Plants were subjected to two, 2-month cold-induced dormancy (CID) periods (indicated by arrows). Positive ELISA values were at least 2× higher than negative controls and were visually rated in intensity on a scale of 1 (least intense) to 4 (most intense). Non-inoculated PPV-CP transgenic plants did not produce positive ELISA readings.

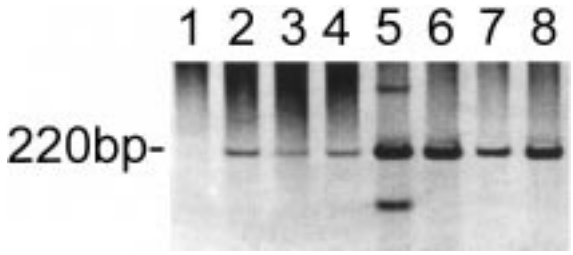

Figure 2. IC-RT-PCR analysis of transgenic and non-transgenic plums indicating the presence of PPV infection: lane 1 – non-inoculated non-transformed control. The remaining lanes are inoculated: Lane 2 – C5 plant 1; lane 3 – C5 plant 2; lane 4 – C5 plant 3; lane 5 – C3 plant 1; lane 6 – C3 plant 2; lane 7 – C2; lane 8 – non-transformed control. PPV-specific primers to the 3′ non-coding region produced a 220 bp product.

Transfer of PPV from ELISA-negative plants to susceptible controls

Although inoculated C5 plants were negative by ELISA, IC-RT-PCR assays were conducted to further evaluate PPV infection in C5. IC-RT-PCR indicated the presence of PPV in these C5 plants (Figure 2, lanes 2–4) as well as in inoculated susceptible PPV-CP transgenic controls (Figure 2, lanes 5–7) and an untransformed control (lane 8). Further, buds of non-transformed controls grafted onto C5 plants in July produced shoots that were PPV ELISA positive and symptomatic in October, indicating that intact, infectious virus particles had moved from these C5 plants into the healthy controls that had been grafted onto them.

PTGS and PPV-CP transgene transcription

To determine if the reduced levels of PPV-CP mRNA in clone C5 (Scorza et al., 1994) were due to post-transcriptional regulation of the transgene, nuclear run-on transcription analysis was performed. Clone C5 (low levels of PPV-CP mRNA), clone C4 (high levels of PPV-CP mRNA), and a non-transformed control were analyzed. Transcription of PPV-CP (Figure 3, all lanes 2) was compared with the transcription of actin (Figure 3, all lanes 1). The non-transformed control produced the actin transcript but not the PPV-CP transcript, as was expected. The levels of both actin and PPV-CP transcripts were at similarly high levels in clones C4 and C5, indicating that the low level of PPV-CP mRNA that accumulated in C5 was due to post-transcriptional down-regulation.

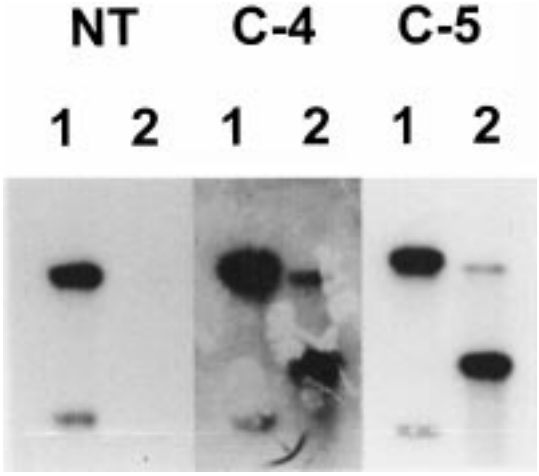

Figure 3. Nuclear run-on assay of non-inoculated PPV-CP transgenic clones C4 and C5. Labeled nuclear RNA was extracted from C4 (susceptible, high level of PPV-CP RNA and CP) and C5 (low PPV-CP RNA, no detectable CP) (Scorza et al., 1994). NT lane is non-transformed control clone 70146. Lane $1 = 2\,\mu g$ of actin plasmid digested by *Xho*l (DNA insert, 0.8 kbp). Lane $2 = 2\,\mu g$ of PPV-CP plasmid double digested by *Bam*Hl and *Asp*718 (DNA insert, 1.2 kbp). The positive reaction in the upper band resulted from partial digestion of the plasmid probes.

PTGS transgene structure in clone C5

In order to investigate the structure of the PPV-CP insert (Ravelonandro et al., 1992) in clone C5, DNA was digested with the restriction enzymes *Eco*RI, *Hin*dIII, and *Bam*Hl. DNA blots were hybridized with the 1.0 kbp fragment from the *PPV-CP* gene, the 1.1 kbp fragment from the *nptII* gene, and the 0.8 kbp fragment from the *gus*A gene (Figure 4). Assuming a complete border to border T-DNA integration of the PPV-CP cassette, an *Eco*RI digestion probed with either *gus*A or PPV-CP sequences would reveal a 7 kbp fragment (Figure 4). An *Eco*RI digest probed with the PPV-CP sequence would produce a fragment of at least 3 kbp resulting from a cut outside of the right border of the insert. This fragment length and number would be variable depending on the location and number of inserts. The *npt*II probe also would hybridize to this > 3 kbp fragment. When the *Eco*RI digestion of C5 DNA was hybridized with the PPV-CP sequence five fragments were produced: a 5 and 10 kbp fragment that corresponded to the predicted > 3 kbp fragment (these two fragments also hybridized to the *npt*II probe as predicted), and the predicted 7 kbp fragment in common with *gus*A (Figure 5 lane 1). In addition to these three predicted fragments, two fragments unique to the PPV-CP probe were observed at 3 and 1.9 kbp (Figure 5, lane 1). These last two fragments would not be expected if complete duplication of the PPV-CP cassette with intact *Eco*RI sites had occurred. These fragments may be explained by a separate insertion or a duplication of the *PPV-CP* gene insert that is unlinked to the *nptII* and *gus*A gene inserts. Hybridization with the *npt*II probe revealed a unique 20 kbp fragment that did not hybridize with either the *gus*A or the PPV-CP probe (Figure 5, lane 2). This fragment may have resulted from a separate insertion or duplication of the *nptII* gene. Hybridization of the *Eco*RI digest with the *gus*A probe showed, in addition to the expected 7 kbp fragment, a smaller than predicted 5 kbp fragment which could have resulted from a truncation of the *gus*A gene (Figure 5, lane 3). Apparent truncation of the *gus*A gene at the left border of the insert has previously been reported using this particular T-DNA vector (Scorza et al., 1995).

When hybridized with the PPV-CP probe, blots of *Hin*dIII-digested DNA revealed the expected 2 kbp fragment, and *Bam*Hl-digested DNA showed the expected 1.2 kbp PPV-CP fragment (see map in Figure 4). However, larger fragments also hybridized with the PPV-CP probe. One of these larger than expected fragments following digestion with *Hin*dIII can be seen in Figure 6, lane 1. The large fragments are possibly the result of restriction site methylation, mutation, deletion, or rearrangement of the restriction site.

Transgene methylation in clone C5

In order to evaluate methylation of the transgene insert in clone C5, DNA was digested with methylation sensitive (*Alu*I, *Bam*HI, *Hin*dIII, *Sau*3A) and methylation insensitive (*Mbo*I, *Hin*fI) enzymes. The digests were split into four subsamples and hybridized with probes made to the *PPV-CP*, *nptII*, *gus*A genes and to the CaMV 35S promoter region. The same digests were also subjected to PCR, following the strategy of Ingelbrecht et al. (1994), using the primers that were used to construct the probe sequences. Figure 6 presents the results from one such experiment using DNA from the silenced C5 and the non-silenced C3 lines. The DNA was initially digested with *Hin*dIII and then with either *Mbo*I or *Sau*3A. Evidence of methylation is seen in the PPV-CP sequence in C5. This is indicated by the multiple, larger than expected-sized fragments in the *Sau3*A digest as compared to the *Mbo*I digest when probed with PPV-CP (Figure 6 PPV-CP, lane 3). No

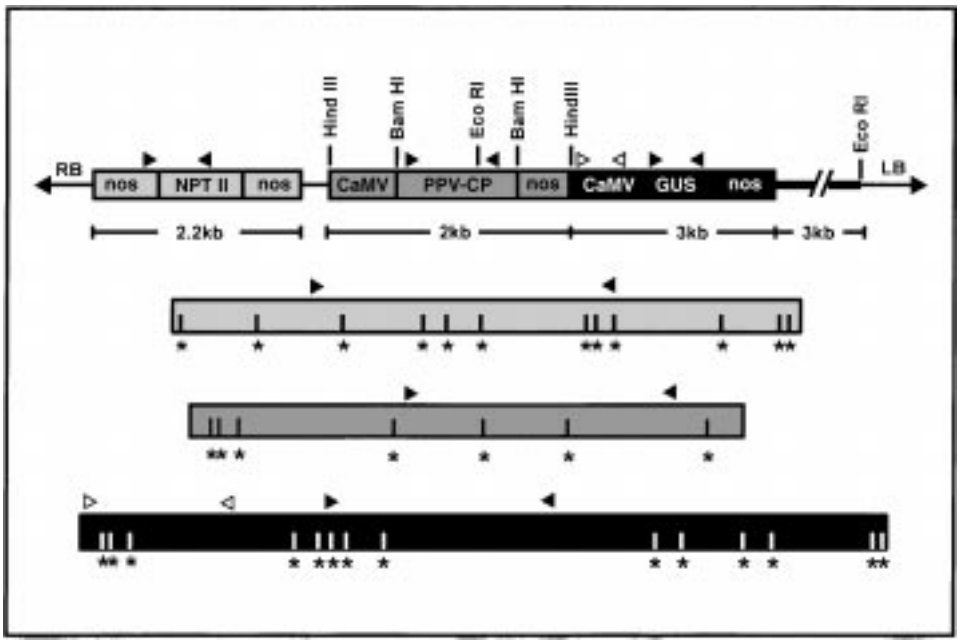

Figure 4. Schematic diagram of the structure of the cassette used for plum transformation with PPV-CP, 20% gray indicates *npt*II, 60% gray = PPV-CP, black = *gus*A. Asterisks indicate *Mbo*I, *Sau*3A isoschizomer restriction sites. Open arrows indicate primer locations for CaMV 35S promoter, black arrows = primer locations for indicated coding regions. The *Bam*HI-isolated PPV-CP fragment is 1.2 kbp.

differences were seen in C3 between the *Mbo*I and *Sau*3A digests (Figure 6 all lanes 5 and 6) nor in the C5 when hybridized with *gus*A, *npt*II or CaMV 35S sequences (Figure 6, lanes 2 and 3 of the respective probes). These results indicated specific methylation of the PPV-CP insert, lack of methylation of the *gus*A, *npt*II and CaMV 35S sequences, and complete digestion of the sampled DNA. The PCR reactions for the same digests (Figure 6) supported the DNA blotting indicating that the C5 PPV-CP but not the *npt*II or the *gus*A transgenes were methylated since PCR of the *Sau*3A digest using PPV-CP-specific primers generated product only in clone C5 (Figure 6, PPV-CP lane 3). Additionally, although DNA blotting did not indicate methylation of the CaMV 35S promoter region in C5, PCR results, which are more sensitive than DNA blotting, indicated a low level of methylation in C5 (Figure 6, CaMV 35S PCR lane 3).

Methylation of the PPV-CP insert in C5 was further investigated by digesting C5 and C3 DNA with *Bam*H1 and then with the C-methylation sensitive enzymes *Alu*I, *Eco*RI, and *Sau*3A, and methylation insensitive *Hin*fl. Comparison of the C3 and C5 digests reveals the presence of multiple, larger fragments in C5 indicating C-residue methylation (Figure 7, lanes 1, 3, 4). Although, unlike methylation sensitive *Alu*I, *Sau*3A, and *Eco*RI digestions, the unexpected fragment from the *Hin*fl digestion cannot be explained by methylation. This unexpected fragment is likely the result of rearrangement or other aberrations of the PPV-CP insert in C5 (Figure 7, lane 2).

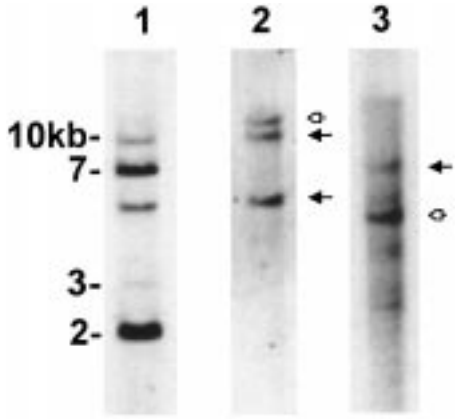

Figure 5. DNA blot analysis of transgenic clone C5 genomic DNA digested with *Eco*RI. Lane 1 was hybridized with a 1.0 kbp PCR-generated PPV-CP fragment; lane 2 – hybridized with a 1.1 kbp PCR-generated *npt*II fragment; lane 3 – hybridized with a 0.8 kbp PCR-generated *gus*A fragment. Solid arrows indicate fragments in common with PPV-CP hybridization, open arrows indicate unique fragments. Fragment sizes indicated on left were derived from molecular weight standards.

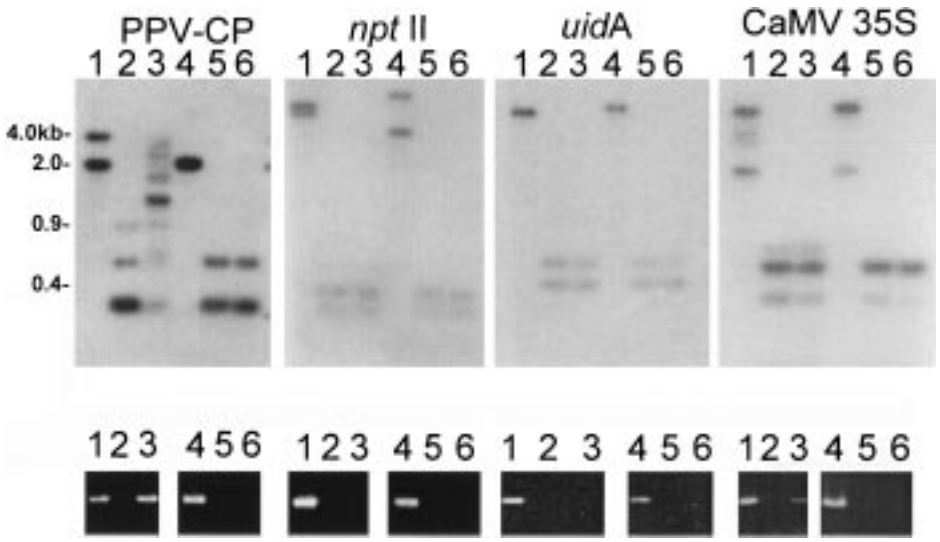

Figure 6. DNA blot and PCR analysis of genomic DNA from transgenic clones C5 (silenced) and C3 (non-silenced) demonstrating methylation status of the *PPV-CP*, *nptII*, and *gus*A genes, and CaMV 35S promoter. Lanes 1–3 are C5. Lanes 4–6 are C3. Lanes 1 and 4 were digested with *Hin*dIII, lanes 2 and 5 with *Hin*dIII and *Mbo*I. Lanes 3 and 6 were digested with *Hin*dIII and *Sau*3A. Hybridization probes and primer pairs from left to right: PPV-CP, *npt*II, *gus*A, CaMV 35S. Fragment sizes indicated on left were derived from molecular weight standards.

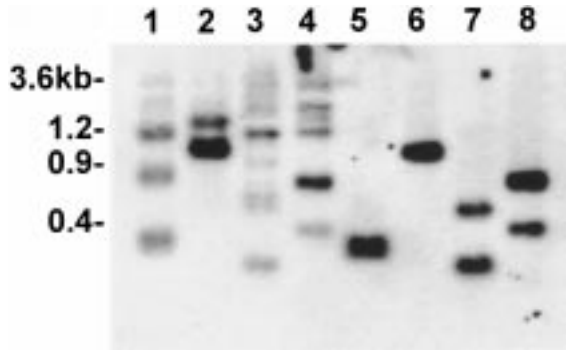

Figure 7. DNA blot analysis of genomic DNA from transgenic clones C5 and C3 demonstrating methylation of the PPV-CP gene insert: Lane 1 – double-digestion of C5 with *Bam*HI and *Alu*I; lane 2 – double-digestion of C5 with *Bam*HI and *Hin*fI; lane 3 – double-digestion of C5 with *Bam*HI and *Sau*3A; lane 4 – double-digestion of C5 with *Bam*HI and *Eco*RI; lanes 5–8 – C3 digested with the same restriction enzymes as in lanes 1–4, respectively. Fragment sizes indicated on left were derived from molecular weight standards. *Alu*I, *Bam*HI, *Eco*RI, and *Sau*3A are methylation sensitive; *Hin*fI is methylation insensitive. Blots were hybridized with the 1.0 kbp PPV-CP-specific probe.

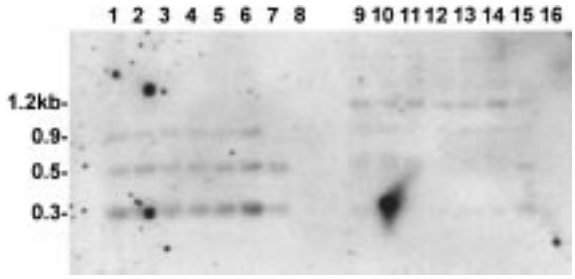

Figure 8. DNA blot analysis of C5 progeny genomic DNA showing methylation of *PPV-CP* gene insert: Lanes 1–8 – double-digestion with *Bam*HI and *Mbo*I (methylation insensitive); lanes 9–16 – double-digestion with *Bam*HI and *Sau*3A (methylation sensitive); lanes 1 and 9, C5 parent. Lanes 2–6 and lanes 10–14 are five seedling progeny of C5; lanes 7 and 15, transgenic clone C3. Lanes 8 and 16 are non-transformed control. Fragment sizes indicated on left were derived from molecular weight standards.

PPV-CP transgene methylation in C5 progeny

Non-PPV-inoculated progeny of hybridizations between clone C5 and susceptible plum lines (Scorza et al., 1998) were assayed for methylation by hybridizing restriction digests (double digests of *Bam*HI and *Mbo*I or *Bam*HI and *Sau*3A) of C5 and C3 DNA with the PPV-CP probe previously described. These digests indicated that the PPV-CP insert in C5 progeny was methylated (Figure 8, lanes 1–5 and 9–13) as it was in the parental C5 clone (Figure 8, lanes 6 and 14). Clone C3 did not show evidence of methylation (Figure 8, lanes 7 and 15).

Previous studies on bud-graft-inoculated plants of the same progeny indicated that after 11 months these plants were PPV ELISA negative (Scorza et al., 1998). In the current study these inoculated plants remained symptomless and ELISA negative for up to 29 months.

Discussion

Virus resistance in transgenic plants mediated by the PTGS mechanism has been widely reported in herbaceous species. Our study with plum is unique in demonstrating not only that PTGS occurs in a woody perennial species but that PTGS confers virus resistance to an economically important woody perennial fruit crop. Many of the hallmarks of PTGS as described in herbaceous species were observed in the PTGS transgenic plum line C5. These characteristics included a high level of virus resistance, a high level of transgene transcription in the nucleus, low levels of transgene mRNA in the cytoplasm, a complex multicopy transgene insertion with aberrant copies, and transgene methylation.

Analysis of methylation indicated that the PPV-CP transgene is specifically methylated in contrast to the *gus*A and *npt*II transgenes even though multiple copies of these genes are present. There appears to be a low level of methylation in at least one of the CaMV 35S promoter regions as detected by PCR. Since one CaMV 35S promoter drives expression of the methylated *PPV-CP* gene, methylation may have spread from this gene into the promoter. While the PPV-CP insert is multicopy, aberrant copies of this gene also appear to be present in clone C5. These copies may be a key factor in methylation and PTGS (Stam et al., 1997; Waterhouse et al., 1998; Kohli et al., 1999). The precise nature of the aberrant fragments is currently under study.

Grafting experiments indicated that although PPV was not detectable by ELISA in C5 plants, a low level of virus was present as detected by IC-RT-PCR. Low-level virus accumulation in apparently healthy, silenced transgenic plants has been reported by Guo et al. (1999). Virus in C5 was transported through the graft union where it proliferated in non-transformed, susceptible shoots that had been bud-grafted onto the inoculated C5 plants. It is important to note however that the transgenic C5 plants were under intense and continuous infection pressure not only from the original inoculum shoots that were growing on these plants, but also from infected rootstocks onto which they were grafted. PPV has been shown to move into the roots of plum trees soon after infection (Adams & Patterson, 1980), and roots have been shown to remain highly infected throughout the growing and dormant seasons (Adams et al., 1998). This presents a consideration for the use of PTGS in woody perennial crops. Most woody perennial tree fruits are propagated through grafting which is a process based on forming direct connections through the vascular system of rootstock and scion. If PTGS virus-resistant clones are grafted onto virus-infected rootstocks, the PTGS scions could carry a low level of virus. In practice, it might be necessary to use not only PTGS virus resistant scions but also PTGS rootstocks. Alternately, PTGS scion varieties could be grown on their own roots. In the absence of infection through graft transmission, studies by Malinowski et al. (1998) showing that C5 plants exposed to natural aphid vectored PPV have remained virus-free for 2 years (including 2 periods of natural CID) suggest that this PTGS clone may be immune to natural aphid-vectored PPV infection.

We have previously shown that the complex multicopy transgene insert in PPV-resistant clone C5 was inherited as a single block of genes and that resistance is correlated with the presence of the transgene insert in the progeny (Scorza et al., 1998). We show in the present study that the PPV-CP insert in non-inoculated progeny was methylated. It is not clear how early in the development of the seedlings methylation occurred since assays were performed over one year following seed germination. PPV resistance in C5 progeny demonstrates the potential for use of C5 as a parent in breeding virus resistant plum lines and thereby provides a novel source of germplasm for breeding new, plum pox resistant plum rootstock and scion varieties.

Our results show a close relationship between virus resistance, methylation, and PTGS, confirming the work of Jones et al. (1999) and others (English et al., 1996; Sijen et al., 1996). Further, we extend these findings to woody perennials and show the potential value of this technology for the development of virus resistant fruit trees.

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